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Systems Biology Analyses Reveal Hyperactivation of Transforming Growth Factor beta and JNK Signaling pathways in Esophageal Cancer

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Abstract

Background & Aims: Esophageal adenocarcinoma (EAC) is resistant to standard chemoradiation treatments and few targeted therapies are available. We used large-scale tissue profiling and pharmacogenetic analyses to identify deregulated signaling pathways in EAC tissues that might be targeted to slow tumor growth or progression.

Methods: We collected 397 biopsy specimens from patients with EAC and non-malignant Barrett's esophagus (BE), with or without dysplasia. We performed RNA sequencing analyses and used systems biology approaches to identify pathways that are differentially activated in EAC vs non-malignant dysplastic tissues; pathway activities were confirmed using immunohistochemistry and quantitative real-time PCR analyses of signaling components in patient tissue samples. Human EAC (FLO-1 and EsoAd1), dysplastic BE (CP-B, CP-C, CP-D), and non-dysplastic BE (CP-A) cells were incubated with pharmacologic inhibitors or transfected with small interfering RNAs. We measured effects on proliferation, colony formation, migration, and/or growth of xenograft tumors in nude mice.

Results: Comparisons of EAC vs non-dysplastic BE tissues revealed hyperactivation of transforming growth factor beta (TGFB) and/or Jun N-terminal kinase (JNK) signaling pathways in more than 80% of EAC samples. Immunohistochemical analyses demonstrated increased nuclear localization of phosphorylated JUN and SMAD proteins in EAC tumor tissues compared to non-malignant tissues. Genes regulated by TGFB and JNK pathway were overexpressed specifically in EAC and dysplastic BE. Pharmacologic inhibition or knockdown of TGFB or JNK signaling components in EAC cells (FLO-1 or FLO-1 or EsoAd1) significantly reduced cell proliferation, colony formation, cell migration, and/or growth of xenograft tumors in mice, in a SMAD4-independent manner. Inhibition of the TGFB pathway in BE cell lines reduced proliferation of dysplastic but not non-dysplastic cells.

Conclusions: In a transcriptome analysis of EAC and non-dysplastic BE tissues, we found TGFB and JNK signaling pathways to be hyperactivated in EACs, and the genes regulated by these pathways to be overexpressed in EAC and dysplastic BE. Inhibiting these pathways in EAC cells reduces their proliferation, migration, and formation of xenograft tumors. Strategies to block the TGFB and JNK signaling pathways might be developed for treatment of EAC.

Graphical Abstract

Keywords

PARADIGM; InFlo; LY2157299; SP600125

Over the last few decades, the incidence of esophageal adenocarcinoma (EAC) has more than quadrupled, especially among Caucasian men in the United States^{1, 2} While most EACs arise in Barrett's esophagus (BE)³, a replacement of normal esophageal squamous epithelium with metaplastic columnar mucosa, the majority of patients with BE patients do not develop dysplasia or cancer in their life-time³. Nonetheless, once BE progresses to advanced or unresectable EAC, treatment options are limited, and 5-year survival is <20%⁴ Deciphering the molecular underpinnings of EAC pathogenesis could thus enable development of new targeted-therapeutic strategies for this currently refractory cancer.

Here, we employed systems biology approaches to analyze global transcriptomic profiles derived from treatment-naïve BE-associated malignant and non-malignant tissues, enabling us to identify deregulations in signaling pathway activities on a genome-scale, along the BE-to-EAC continuum. Subsequent pharmacogenetic studies on selected EAC-associated activated pathways revealed novel molecular dependencies and new therapeutic targets for this lethal cancer.

MATERIALS AND METHODS

Detailed methods are provided as Supplementary Methods.

Patient samples

Study subjects were recruited and informed consent was obtained under the University Hospitals Institutional Review Board approved protocol (UHCMC IRB # CC301), as part of the Barrett's Esophagus Translational Research Network (BETRNet). Tissue was collected from patients with Barrett's esophagus (BE) undergoing routine surveillance and patients with newly diagnosed esophageal adenocarcinoma (EAC) undergoing endoscopic procedures. Endoscopic biopsies were immediately snap frozen at bedside to preserve RNA. Biopsies from patients with non-dysplastic stable BE (NDSBE) or high-grade dysplasia (HGD) were obtained preferentially from areas likely to have intestinal metaplasia or high-grade dysplasia, respectively, using high definition white light and narrow band imaging guidance. NDSBE biopsies were obtained from subjects with at least 2 cm of BE who had no history of dysplasia and have not developed dysplasia or cancer (median surveillance of 9 years, ranging from 6 to 22 years). Biopsies from cancer were obtained preferentially from areas of visible cancer that did not appear ulcerated or necrotic at endoscopy. Paired, normal (uninvolved) esophageal squamous tissue (SQ) from each of EAC cases were obtained a minimum of 3 cm proximal to the squamo-columnar junction. Gastric biopsies (GAST) were obtained from the gastric fundus in patients with NDSBE or EAC. In each case, sister biopsies, obtained adjacent to the frozen biopsies, were formalin-fixed and paraffin-embedded for subsequent histological examination by an expert anatomic pathologist (J.E.W.) to confirm the diagnosis. The majority of patients included in the study are Caucasian males. Taken together, the discovery cohort consisted of 105 samples including

18 NDSBEs, 56 pre-treatment EACs, 20 normal SQ samples matching respective EACs, and 11 normal GAST samples. To confirm the candidate pathway alterations identified in the discovery set, we compiled an independent validation cohort consisting of 292 biopsy samples, including 48 pre-treatment EACs, 21 HGDs, 64 Barrett's metaplasia (BM) (for whom follow-up was unavailable), and 159 normal SQ samples.

Estimation of pathway activities using systems biology approaches

RNA sequencing (RNAseq) was performed on patient biopsy tissues as previously described by our group ⁵. PARADIGM modeling approach ⁶ was used to identify differentially activated pathways in EACs, and findings were further validated using an independent computational framework (InFlo) that we recently developed ⁷. Selected differentially-activated pathways in EACs were further confirmed using both unsupervised and supervised analyses of pathway-specific gene expression modules. Activities of the selected pathways were additionally evaluated using immunohistochemistry (IHC) analysis of paradigmatic pathway-specific protein readouts in formalin-fixed paraffin-embedded (FFPE) tissue sections, and by quantitative real-time PCR (qPCR) analysis of expression of pathway-specific target-genes in clinical tissues.

Cell culture

Human EAC cell line, FLO-1, was kindly provided by Dr. Navtej Buttar (Mayo Clinic, Rochester, MN) and EsoAd1 by Drs. James Eshleman and Anirban Maitra (Johns Hopkins Medical Institutions, Baltimore, MD). Non-dysplastic (CP-A) and dysplastic (CP-B, CP-C, CP-C) human BE cell lines (American Type Culture Collection, Manassas, VA) were cultured as previously described ⁸. Vaco400 and Vaco8-2 microsatellite stable colon cancer cell lines were previously generated by our group ⁹. Cell lines were obtained in 2014 (except Vaco400 and Vaco8-2), and tested for authenticity using STR genotyping, and were screened periodically for mycoplasma contamination.

In vitro phenotypic characterization using EAC cell line models

Cell growth assessments were quantified following seeding using the IncuCyte ZOOM automated live cell kinetic imaging system (Essen BioScience), as previously described by our group ⁵. Colony growth assessments were quantified as previously described by our group ¹⁰. Significant differences in cell growth/clonogenicity between test *versus* control groups were estimated using a one-sided Student's t-test assuming unequal variances.

In vivo tumor xenograft growth assays

Tumor xenografts were induced by subcutaneous injections of FLO-1 or EsoAd1 cells (4×10^6), suspended in 50% Geltrex, bilaterally into the flanks of 4–5 week old female athymic Foxn1 nu/nu mice. After the xenograft tumors reached a minimum size of 50mm³, mice were randomized and treated with either vehicle, or with 50 mg/kg SP600125 or SB431542 (via i.p), or with 100 mg/kg LY2157299 (via oral gavage), and followed longitudinally. Significant differences in tumor volumes between the respective treatment *versus* vehicle-control groups were estimated using a Student's t-test assuming unequal

variances. All animal procedures were approved by the Case Western Reserve University Institutional Animal Care and Use Committee and followed NIH guidelines.

RESULTS

Systems biology analyses identify hyperactivation of JNK–JUN and TGFβ–SMAD signaling in the majority of EACs

We performed RNAseq on a discovery cohort ($N=105$) consisting of 56 pre-treatment EACs, 18 samples of never-dysplastic Barrett's metaplasia (NDSBE), 20 samples of normal esophageal squamous (SQ), and 11 normal gastric biopsy tissues (Supplementary Table S1). RNAseq profiles demonstrated a striking tissue-specific clustering of samples (Supplementary Figure S1), suggesting these transcriptomic profiles capture molecular alterations associated with esophageal tumor pathogenesis. Employing state-of-the-art pathway modeling frameworks, PARADIGM and InFlo^{6,7}, with non-malignant NDSBE as the histological baseline comparator, we identified three major pathway sub-networks that demonstrate selective hyperactivation in EACs (Figure 1A). Of these, JNK–JUN and TGFβ–SMAD sub-networks were of particular interest due to their significant roles in other cancer types. As compared to NDSBE, hyperactivation of JNK–JUN and TGFβ–SMAD sub-networks was observed in 79% and 73% of EACs, respectively (Figure 1B). 68% of EACs showed hyperactivation of both sub-networks, and 84% showed activation of at least one of these sub-networks (Supplementary Figure S2).

To verify these computational predictions, we tested primary EAC tissues for induction of protein phosphorylation and target-gene expression associated with active JNK–JUN and TGFβ–SMAD signaling. Immunohistochemical (IHC) analysis of phosphorylated c-JUN and SMAD proteins, key functional readouts of JNK–JUN and TGFβ–SMAD pathway activation, revealed increased induction and nuclear localization of these proteins in primary EAC tumors as compared to NDSBE (Figure 2, A and B; Supplementary Figure S3). We however note that despite their high signal-specificities in Western Blots, phospho-SMAD/-JUN antibodies are not ideal or indicated for IHC-based assessments. We therefore suggest using caution when interpreting IHC-based signal readouts of these anti-phosphoprotein antibodies under FFPE conditions.

Unsupervised analysis of known JUN and SMAD transcriptional target-genes (Supplementary Tables S2 and S3) revealed distinct tissue-specific clusters (Figure 2, C and D), while supervised analyses revealed a significant fraction of both JUN and SMAD target-genes as being overexpressed in EACs (Supplementary Figure S4). Quantitative real-time PCR (qPCR) analysis of representative JUN and SMAD target-genes confirmed their >8-fold overexpression in primary EACs versus NDSBEs ($P < 10^{-5}$, Figure 2, E and F).

To test the generalizability of these findings, we examined expression of JUN and SMAD target-genes in an independent validation sample-set of pre-treatment EAC and non-malignant biopsy tissues ($N=292$, Supplementary Table S4). qPCR again demonstrated >10-fold overexpression of JUN and SMAD target-genes in EACs versus Barrett's metaplasia ($P < 10^{-5}$, Figure 2, G and H). Notably, up-regulation of JUN and SMAD target-genes was also evident in high-grade dysplasia (HGD) lesions included in the validation cohort (Figure 2, G

and H), suggesting that hyperactivation of these signaling pathways may occur at the early dysplastic phase of BE progression. Taken together, our findings of hyperactive JNK–JUN and TGFβ–SMAD signaling preferentially in EAC and HGD lesions suggested that these pathways could be drivers of tumor progression.

Pharmacogenetic assessments reveal pro-tumorigenic effects of TGFβ–SMAD and JNK–JUN signaling activation in EACs

Given the hyperactivation of TGFβ/JNK signaling networks in the majority of EACs (Figure 1), we tested for potential oncogenic activities of these pathways in EAC, using the well-characterized FLO-1 EAC cell line model, wherein RNAseq-based analysis showed hyperactivation of both these signaling networks (Supplementary Figure S5). First, we assessed the effect of TGFβ1 ligand on the growth of FLO-1 EAC cells. While TGFβ1 exhibited no effects on FLO-1 cells when grown in bulk culture (Supplementary Figure S6), TGFβ1 significantly enhanced the growth and colony forming ability of FLO-1 cells when sparsely seeded in soft agar (Figure 3A). We next tested whether inhibiting signaling downstream of TGFβ ligands could suppress FLO-1 growth. Treatment of FLO-1 EAC cells with the TGFβRI kinase inhibitors (SB431542 or LY2157299)^{11–16} caused significant dose-dependent growth reduction of cells in bulk culture (Figure 3, B and C), with concomitant reduction in SMAD phosphorylation and expression of the canonical TGFβ downstream target-gene, SERPINE1 (Figure 3, B and C; Supplementary Figure S7A). Similarly, treating FLO-1 cells with a JNK inhibitor, SP600125, blocked JUN phosphorylation and caused marked dose-dependent suppression of cell growth (Figure 3D).

To further test whether the growth promoting activity of TGFβ and JNK pathways in EACs are mediated by canonical signaling components, we performed knockdown studies against key TGFβ and JNK signaling mediators. For TGFβ pathway, we performed siRNA-based transient knockdown of TGFβR1, TGFβR2, SMAD2, SMAD3, SMAD4 (Supplementary Figure S7B). Additionally, knockdown studies were performed on TGFβ ligands (TGFβ1, TGFβ2, TGFβ3) given their upregulation in primary EAC tissues as well as the FLO-1 cell line, as compared to non-malignant tissues (Supplementary Figure S8). Similar knockdown studies were performed on c-JUN to assess for the pro-tumorigenic activity of the JNK pathway. Knockdown of TGFβR1, TGFβR2, SMAD2, SMAD3, TGFβ1, TGFβ2, TGFβ3, or c-JUN significantly suppressed FLO-1 cell growth (short-term) and colony growth (long-term), while knockdown of SMAD4 showed no such growth-suppressive effects (Figure 3, E and F). All siRNA based knockdown studies were conducted using a pool of four non-overlapping siRNAs per gene, and findings were further confirmed with two individual siRNAs per gene (Supplementary Figure S9). In particular, these findings demonstrate the growth dependency of EACs on TGFβ signaling, while provocatively suggesting that the pro-tumorigenic activity of TGFβ pathway may be independent of SMAD4.

Inhibition of TGFβ–SMAD signaling suppresses growth of SMAD4-null EAC cells

To further test if pro-growth effects of TGFβ–SMAD signaling in EACs are both general and SMAD4-independent (Figure 3), we reprised the above studies in a second EAC cell line model, EsoAd1, that harbors bi-allelic inactivating mutations in SMAD4 (Supplementary Figure S10). As expected, TGFβ1 ligand showed no ability to suppress growth of these

SMAD4-null EAC cells in cell culture (Supplementary Figure S11). Of note, sparsely-seeded EsoAd1 cells in soft agar do not form colonies thus precluding assessment of TGF β 1 effects on growth and colony forming ability of these cells in soft agar. However, in this second and SMAD4-null EAC model, a significant and dose-dependent suppression of cell culture growth was again achieved by blocking TGF β signaling (Figure 4, A and B, Supplementary Figure S12) with small molecule inhibitors (SB431542, LY2157299). We note that, the increased growth suppression observed with SB431542 versus LY2157299 in EsoAd1, but not in FLO-1 (Figure 4, A and B), may reflect EsoAd1's significant expression of ALK7 (Supplementary Figure S13) that is inhibited by SB431542 but not by LY2157299. EsoAd1 cell and colony growth were also significantly reduced by siRNA knockdown of TGF β ligands, TGF β R1, TGF β R2, SMAD2, or SMAD3, thus supporting the involvement of these components in a SMAD4-independent growth promoting limb of TGF β signaling (Figure 4C). EsoAd1 also resembled FLO-1 cells in being additionally dependent on JNK signaling, and in demonstrating growth suppression following inhibition of JNK signaling (Supplementary Figure S14). All siRNA based knockdown studies were conducted using a pool of four non-overlapping siRNAs per gene, and findings were further confirmed with two individual siRNAs per gene (Supplementary Figure S15). In addition, inhibition of TGF β or JNK signaling also markedly reduced EsoAd1 cell's migratory potential (Supplementary Figure S16).

Inhibition of TGF β –SMAD signaling ablates EAC growth *in vivo*.

To test the TGF β pathway as a potential therapeutic target in EAC, we established EsoAd1 and FLO-1 tumor xenografts in immune-deficient mice. Reprising our findings in primary EAC tissue (Figure 2), IHC analyses of xenograft sections demonstrated increased expression and nuclear localization of phospho-SMAD proteins in the SMAD4-null EsoAd1 as well as FLO-1 tumors (Figure 5; Supplementary Figures S17 and S18).

Tumor xenografts were allowed to grow till they were established and had reached a minimum size of 50mm³, when mice were then randomized to start treatment with TGF β R1 inhibitors (SB431542, *i.p.*, LY2157299, *oral gavage*), or with vehicles only (see Methods). EsoAd1 tumors proved strikingly sensitive to inhibition of TGF β signaling, with either SB431542 or LY2157299 treatment abrogating the growth of and eradicating these SMAD4-null EsoAd1 tumors (Figure 5A), with none of the TGF β R1 inhibitor treated mice showing any tumor recurrence during 21 days of post-treatment follow-up. Similarly, inhibiting TGF β signaling proved highly potent in suppressing the growth of FLO-1 EAC tumors (Figure 5B). In particular, mice treated with the LY2157299 TGF β R1 inhibitor showed a near total regression of FLO-1 tumor growth *in vivo* (Figure 5B). Taken together, these two models suggest that active TGF β –SMAD signaling contributes to and sustains EAC cell growth *in vivo*. Parallel studies also demonstrated dependence of *in vivo* growth of these two EAC models on activated JNK signaling. In particular, in both tumor models, IHC analysis showed the presence and nuclear localization of phospho-c-JUN protein and treating xenograft-bearing mice with the JNK inhibitor SP600125 abrogated tumor growth (Figure 5, C and D; Supplementary Figures S17 and S18). Of note, none of the inhibitors at indicated doses induced any detectable adverse effects on mice or on their body weights throughout the study duration (Supplementary Figure S19).

Reversal of TGF β –SMAD signaling from growth suppressor to promoter correlates with progression to esophageal dysplasia

We next evaluated whether pro-tumorigenic effects of the TGF β –SMAD signaling are restricted to the malignant EAC stage, or whether they develop earlier in pre-malignant Barrett's metaplasia or dysplasia, that are early and late precursors of EAC. We tested effects of stimulation or inhibition of TGF β –SMAD signaling in four available and well-characterized BE cell line models. While TGF β 1 ligand treatment markedly suppressed the growth of non-dysplastic BE cells (CP-A) in a SMAD4-dependent manner (Figure 6A, Supplementary Figure S20), it failed to inhibit the growth of any of the dysplastic BE cell lines tested (CP-B, CP-C, CP-D; Figure 6A). Conversely, inhibiting TGF β –SMAD signaling (Figure 6B, Supplementary Figure S21) with SB431542 or LY2157299 compounds suppressed the growth of two of the three dysplastic BE cell lines (CP-B, CP-D), but had no effects on growth of the non-dysplastic BE cells (Figure 6B). Furthermore, siRNA mediated knockdown of TGF β ligands/receptors/SMAD components showed that reducing SMAD2 or SMAD3 exclusively induced significant growth suppression in the dysplastic BE cells (Supplementary Figure S22). Of note, knockdown of TGF β 3 ligand resulted in marked suppression of both non-dysplastic and dysplastic BE cell growth (Supplementary Figure S22), likely suggesting a generalized cytotoxic effect of inhibiting TGF β 3. Interrogation of the JNK pathway revealed growth suppression of both non-dysplastic and dysplastic BE cells when treated with either chemical or siRNA inhibitors of JNK (Supplementary Figure S23). Overall, these findings reveal the differential dependencies of dysplastic versus non-dysplastic BE cell growth on TGF β signaling, and point to the potential pro-tumorigenic role of SMAD components in pre-malignant phases of the disease. Furthermore, these findings additionally suggest that TGF β –SMAD signaling may switch from being a growth suppressor to being a growth promoter as early as in Barrett's dysplasia, and may thus contribute to the progression of BE dysplasia to frank invasive EAC.

DISCUSSION

In summary, our application of systems biology approaches ^{6, 7} to infer pathway activities from primary tissue transcriptome profiles, combined with pharmacogenetic analyses, identified TGF β and JNK signaling pathways as potential oncogenic promoters in Barrett's-associated neoplasia.

Contrary to prior suggestions of TGF β signaling playing a tumor suppressor role in Barrett's neoplasia ^{17–20}, our findings unexpectedly point to TGF β as being a tumor promoter in EAC, with the pro-tumorigenic effects of this pathway evident as early as the dysplastic phases of disease progression. Intriguingly, this paradoxical growth promoting effect of the TGF β pathway in EAC/dysplastic BE cells appears to be mediated via a SMAD4-independent, but seemingly SMAD2/3 dependent arm of the TGF β signaling pathway. These observed growth-inhibitory (in non-dysplastic BE) versus growth-promoting (in EAC/dysplastic BE cells) effects of TGF β pathway were not associated with its non-canonical ²¹ regulation of MAPK/ERK signaling (Supplementary Figure S24). Nonetheless, given the differences in TP53 mutation status between the non-dysplastic BE (TP53 wild-type) versus dysplastic BE/EAC (TP53 mutant) cells ²², it is possible that the growth-promoting function

of TGF β signaling may be dependent on dysfunctional TP53 in this disease. Future studies are however warranted to test this association and broader significance of genomic alterations such as TP53 mutations as potential modulators of TGF β signaling. Importantly, we additionally note that the growth-promoting effects of TGF β –SMAD signaling in EACs are in direct contrast to the known tumor suppressor function of this pathway in colon adenocarcinomas⁹ In line with this, unlike EACs, treatment of colon adenocarcinoma cells with TGF β 1 markedly suppressed cell growth, while TGF β RI inhibition showed no growth-suppressive effects either in SMAD4 wild-type or SMAD4-null colon adenocarcinoma cells, (Supplementary Figure S25). This functional distinction in the role of TGF β as a potential promoter of EAC, but as a suppressor of colon cancer, is further supported by the genetic observations that somatic mutations in TGF β signaling elements are rare in EACs (~11%)^{23, 24}; whereas, colon cancers commonly demonstrate mutations in the *TGF β RII* gene (~30%)⁹, or mutations (~24%) or genomic deletions (~60%) in SMAD's^{25–27} Collectively, these findings point to fundamental differences in the biologic role of TGF β –SMAD signaling in EAC versus other gastrointestinal malignancies such as colon cancer.

Unlike the unexpected finding of oncogenic activity of TGF β in EAC, our findings of EAC growth dependency on JNK–JUN pathway hyperactivation is consistent with the established pro-tumorigenic properties of this pathway. In line with this, previous studies have shown that transactivation of the JNK pathway facilitates leptin-induced stimulation of proliferation and reduced apoptosis in EAC cells²⁸. Additionally, the growth-suppressive effects of curcumin in EAC cells have been suggested, in part, to be mediated via inhibition of JNK–JUN pathway²⁹ Furthermore, the known pro-tumorigenic effects of bile acids in Barrett's-associated carcinogenesis have been suggested, in part, to be mediated via activation of AP-1 protein complex³⁰. These studies, in conjunction with our findings, strongly indicate a pro-tumorigenic role for this pathway in EAC progression.

As ~70% of EACs in our tissue-based studies showed hyperactivation of both TGF β –SMAD and JNK–JUN pathway sub-networks, and given that these pathways are well known to interact³¹, we further assessed for potential phenotypic synergy between TGF β RI and JNK inhibitors in EAC cells. Combination treatment of TGF β RI inhibitors (SB431542, LY2157299) with JNK inhibitor (SP600125) at low doses resulted in marked growth suppression of SMAD4-null EsoAd1 cells, as compared to either inhibitor treatment alone (**Supplementary Figure S26**). However, this interaction was specific to EsoAd1 cells, and was absent in FLO-1 EAC cells. Moreover, TGF β RI inhibitor treatment showed no effect on reducing c-JUN phosphorylation in EAC cells, with LY2157299 in fact inducing phosphorylation of c-JUN (**Supplementary Figure S26**), a finding consistent with a prior report on this compound³². Conversely, treatment with the JNK inhibitor, SP600125, predominantly reduced the phosphorylation of the linker region within SMAD2 (**Supplementary Figure S26**), again consistent with prior reports of the JNK pathway playing a role in SMAD linker phosphorylation^{33, 34} While these findings hint at a potential benefit of TGF β /JNK combination treatment in specific genomic contexts in EACs, future studies will be required to test the generality and mechanisms underlying such potential context dependent synergy between TGF β and JNK signaling pathways.

In summary, our investigations reveal a strong dependency of EAC growth on active TGF β –SMAD and JNK–JUN signaling, and show an unanticipated reversal of the TGF β pathway from its canonical role as a tumor suppressor to instead an EAC growth promoter in the gastrointestinal tract. Further studies will be required to fully decipher the mechanisms underlying the activation and growth promoting effects of TGF β /JNK signaling in EAC. Nonetheless, these findings may have translational significance, as the TGF β R1 inhibitors are currently being evaluated in human trials as an oral anti-cancer agent. These promising preclinical findings thus suggest a rationale for clinical trials to test the potential activity of this compound in treating EAC, a lethal cancer that is refractory to standard cancer therapies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Majority of EACs exhibit hyperactivation of JNK–JUN and TGF β –SMAD signaling sub-networks.

(A) PARADIGM SuperPathway sub-networks exhibiting differential activity in EAC versus NDSBEs. Pathway components showing significant activity differences between EAC (N=56) and NDSBE (N=18) (Wilcoxon P-Value < 0.05) tissue samples are plotted along with their regulatory relationships in shades of red (up-regulated in EACs) and green (down-regulated in EACs), with darker colors corresponding to lower Wilcoxon P-values (see Data Legend). The size of the node corresponds to the percentage of EACs showing deregulation compared to all NDSBEs (see Data Legend). The three dashed boxes indicate major

pathway sub-networks showing selective hyperactivation in EACs. **(B)** Distribution of JNK–JUN and TGF β –SMAD signaling sub-network activities across EACs and NDSBEs. The distribution of activity levels for EAC (N=56) and NDSBE (N=18) tissue samples are plotted for the JNK–JUN and TGF β –SMAD signaling networks respectively. For each sample, the median activity level of all of the components within the respective sub-networks (A) was calculated and plotted along the X-axis. Activity levels > 0 denote hyperactivity, 0 being neutral and < 0 indicating low activity (X-axis) as estimated by PARADIGM. Significant differences in the proportion of samples showing hyperactivity (>0) of a given sub-network between EAC versus NDSBE groups was evaluated using a Fisher's Exact test. Overall, 79% and 73% of EACs exhibit higher JNK–JUN and TGF β –SMAD sub-network activity, respectively, compared to any NDSBE cases in the discovery cohort (Fisher's exact $P < 0.001$).

Figure 2. Orthogonal validation of JNK–JUN and TGFβ–SMAD signaling activities in primary tissue cohorts.

(A,B) Representative Immunohistochemistry images demonstrating marked nuclear localization and expression of phospho-c-JUN (A) and phospho-SMAD (B) proteins in EAC tumor epithelia, as compared to NDSBE (Scale bar, 100μm). The bar graphs below respective images indicate the IHC-based semi-quantitative index of staining (H-score) in NDSBE (N=5) versus EAC (N=5) primary tissue samples. * ($P < 0.05$) indicates significant differences in H-score index between EAC versus NDSBE, estimated using a non-

parametric Mann-Whitney test. **(C,D)** Heatmaps showing hierarchical clustering of the 105 RNAseq discovery tissue samples using RNAseq expression profiles of JUN **(C)** and SMAD **(D)** target-genes. The heatmap represents the median-subtracted log2 FPKM expression level of individual genes across samples. **(E,F)** qPCR analyses showing significantly increased expression of representative JUN **(E)** and SMAD **(F)** target-genes in the EACs. SQ and GAST samples were included as additional comparators in these qPCR analyses. **(G,H)** qPCR analyses of representative JUN **(G)** and SMAD **(H)** target-gene expression in a validation cohort consisting of 48 EAC, 21 HGD, 64 Barrett's metaplasia (BM) with no longitudinal follow-up, and 159 normal esophageal SQ tissue samples. Y-axis shows the fold-changes in the expression of respective target-genes across samples, relative to NDSBE **(E,F)** or BM **(G,H)** cases. Bold horizontal lines within each tissue-type indicate the median fold-change in expression. ** ($P < 0.01$) and *** ($P < 10^{-5}$) indicate significant differences in gene expression between EAC versus NDSBE/BM, or HGD versus BM, estimated using a Student's t-test assuming unequal variances.

Figure 3. Effects of TGFβ-SMAD and JNK-JUN signaling on the growth of FLO-1 EAC cells. (A) Assessment of FLO-1 colony growth in soft agar. Y-axis in the bar graph shows FLO-1 colony numbers following TGFβ1 (10ng/ml) treatment, expressed as a fraction of colony counts observed in the untreated Parental (P) cells. All data are plotted as mean ± SEM, obtained from at least three independent experiments, each performed in triplicates. Significant differences in colony growth were estimated using a Student's t-test assuming unequal variances. Shown below the bar graph are representative images of colony growth in Parental and TGFβ1 treated FLO-1 cells. Also shown below are Western blot images depicting phospho-SMAD induction at 48hrs following TGFβ1 treatment in FLO-1 cells. (B–D) IncuCyte-based growth assessments of FLO-1 cells treated with varying concentrations of TGFβRI inhibitors, SB431542 or LY2157299 (B,C), or with a JNK inhibitor, SP600125 (D). Y-axes represent the average fold-change in cell confluence values normalized to time zero (X-axes) in respective treatment versus DMSO vehicle controls. Also shown are representative Western blot images depicting the protein levels of total and phospho-SMAD/c-JUN, with β-actin as a loading control, at 48hrs following treatment. (E) IncuCyte-based cell (line graphs, Top Left) and colony growth (bar graphs, Bottom Left)

assessments of FLO-1 cells treated with siRNAs targeting TGF β 1, TGF β 2, TGF β 3, TGF β RI, TGF β R2, SMAD2, SMAD3, or SMAD4. Y-axes in line graphs represent the average fold-change in cell confluence values normalized to time zero (X-axes) in respective treatment versus non-targeting control siRNA groups. Y-axes in bar graphs represent the colony numbers for each treatment siRNA groups, expressed as a fraction of colony counts observed in the non-targeting control siRNA. Protein levels of ligands (bar graph) were quantified by ELISA and receptor/SMAD components by Western Blots, at 48hrs following siRNA treatments. (F) IncuCyte-based cell (line graphs, Top) and colony growth (bar graphs, Bottom) assessments of FLO-1 cells treated with siRNAs targeting c-JUN, along with representative Western blot images depicting the protein levels of total and phospho-c-JUN at 48hrs post-treatment. For growth assays, cells were plated in individual wells in replicates and inhibitor/siRNA treatments were performed in each of these wells independently for subsequent analyses. All data are plotted as mean $\pm$ SEM, obtained from at least six biologic replicate measurements. * ($P < 0.05$), ** ($P < 0.005$), and *** ($P < 0.0005$) indicate significant differences between the respective test versus control groups, estimated using a Student's t-test assuming unequal variances.

Figure 4. Small molecule inhibitors of TGFβ-SMAD signaling blocks the growth of SMAD4-deficient EAC cells.

(A–B) IncuCyte-based growth assessments of SMAD4-null EsoAd1 cells treated with varying concentrations of TGFβRI inhibitors, SB431542 or LY2157299. Y-axes represent the average fold-change in cell confluence values normalized to time zero (X-axes) in respective treatment versus DMSO vehicle control groups. (C) IncuCyte-based cell (line graphs, Top Left) and colony growth (bar graphs, Bottom Left) assessments of EsoAd1 cells treated with siRNAs targeting TGFβ1, TGFβ2, TGFβ3, TGFβR1, TGFβR2, SMAD2,

SMAD3, or SMAD4. Y-axes in line graphs represent the average fold-change in cell confluence values normalized to time zero (X-axes) in respective treatment versus non-targeting control siRNA groups. Y-axes in bar graphs represent the colony numbers for each treatment siRNA groups, expressed as a fraction of colony counts observed in the non-targeting control siRNA. Protein levels of ligands (bar graph) were quantified by ELISA and receptor/SMAD components by Western Blots, at 48hrs following siRNA treatments. For growth assays, cells were plated in individual wells in replicates and inhibitor/siRNA treatments were performed in each of these wells independently for subsequent analyses. All data are plotted as mean $\pm$ SEM, obtained from at least six biologic replicate measurements. * ($P < 0.05$) and *** ($P < 0.0005$) indicate significant differences between the respective test versus control groups, estimated using a Student's t-test assuming unequal variances.

Figure 5. Inhibition of TGF β -SMAD or JNK-JUN signaling blocks the growth of EAC cells *in vivo*.

(A–D) Top row, Immunohistochemistry analyses of phosphorylated SMAD and c-JUN proteins in representative EsoAd1 and FLO-1 xenograft tumors (Scale bars, 100 μ m). (A–D) Middle row, assessment of EsoAd1 and FLO-1 tumor xenograft growth in immune-deficient mice treated with (A,B) TGF β RI inhibitors (SB431542, LY2157299), or with (C,D) JNK inhibitor (SP600125), as compared to respective vehicle control treatments (see Methods). Y-axis depicts average fold-change in tumor volume over time (X-axis) in respective treatment groups, normalized to the first day of treatment (time-point zero on X-axis). Data are plotted as mean $\pm$ SEM. ** ($P < 0.005$) and *** ($P < 0.0005$) indicate significant differences in tumor volume at the final time-point between respective treatment versus vehicle-control groups, estimated using a Student's t-test. The number of established EsoAd1 xenograft tumors available for analysis from respective experimental arms at the beginning of the study were 46 (vehicle; i.p), 45 (vehicle; oral gavage), 26 (SB431542), 26 (LY2157299), and 26 (SP600125). The number of established FLO-1 xenograft tumors available for analysis from respective experimental arms at the beginning of the study were 34 (vehicle; i.p), 32 (vehicle; oral gavage), 29 (SB431542), 24 (LY2157299), and 25 (SP600125). (A–D) Bottom row, representative photographic images of mice with *in situ* xenograft tumors at the final time-point across the treatment arms.

Figure 6. Contrasting effects of TGFβ signaling in non-dysplastic versus dysplastic Barrett's esophagus cells.

(A,B) IncuCyte-based cell growth assessments in non-dysplastic (CP-A) and dysplastic (CP-B, CP-C, CP-D) Barrett's esophagus cells treated with (A) TGFβ1 ligand or untreated (Parental/P), or (B) with varying concentrations of TGFβRI kinase inhibitors, SB431542 (SB) and LY2157299 (LY), or with DMSO vehicle (V). Y-axes in line graphs represent the average fold-change in cell confluence values normalized to time zero (X-axes) in respective treatment versus control groups. Also shown are protein levels of SMAD components quantified by Western Blots at 48hrs post-treatment. For growth assays, cells were plated in individual wells in replicates and inhibitors treatments were performed in each of these wells

independently for subsequent analyses. All data are plotted as mean $\pm$ SEM, obtained from at least six biologic replicate measurements. * ($P < 0.05$) and *** ($P < 0.0005$) indicate significant differences in cell growth at the final time-point between the respective treatment versus control groups, estimated using a Student's t-test assuming unequal variances.